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Identification of a HAD superfamily phosphatase, HdpA, involved in 1,3-dihydroxyacetone production during sugar catabolism in *Corynebacterium glutamicum*

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ABSTRACT

Corynebacterium glutamicum **produces 1,3-dihydroxyacetone (DHA) as metabolite of sugar catabolism but the responsible enzyme is yet to be identified. Using a transposon mutant library, the gene *hdpA* (*cgR_2128*) was shown to encode a haloacid dehalogenase superfamily member that catalyzes dephosphorylation of dihydroxyacetone phosphate to produce DHA. Inactivation of *hdpA* led to a drastic decrease in DHA production from each of glucose, fructose, and sucrose, indicating that HdpA is the main enzyme responsible for DHA production from sugars in *C. glutamicum*. Confirmation of DHA production via dihydroxyacetone phosphatase finally confirms a long-speculated route through which bacteria produce DHA.**

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1. Introduction

DHA, the simplest ketose that is composed of three carbon atoms, is a microbial metabolite frequently observed in culture media. Two biochemical reactions for DHA synthesis have been established [1]. The first involves the oxidation of glycerol to DHA by the action of glycerol dehydrogenase. In *Gluconobacter oxydans*, a membrane-bounded glycerol dehydrogenase catalyzes the DHA formation reaction on the plasma membrane and the formed DHA releases into culture medium [2]. The second reaction occurs in a xylulose monophosphate pathway of methylotrophic yeasts and bacteria where dihydroxyacetone synthase catalyzes the conversion of formaldehyde and xylulose 5-phosphate to DHA and glyceraldehyde 3-phosphate [3]. The resulting DHA is basically phosphorylated to form dihydroxyacetone phosphate (DHAP) that is further metabolized for the synthesis of cell constituents and for the regeneration of xylulose 5-phosphate, but the DHA can be excreted into culture medium in DHA kinase-impaired mutants [4].

In addition to the DHA produced from the metabolism of glycerol and C1 compounds, some microorganisms also produce DHA during sugar metabolism. *Zymomonas mobilis*, a well-known ethanol-producing bacterium, anaerobically metabolizes glucose via the Entner Doudoroff (ED) pathway to produce ethanol at high yields (95% of theoretical maximum), but ferments fructose to lower ethanol yields (cf. 90%) primarily due to the formation of by-products including DHA [5]. Based on studies on enzyme activities and metabolites analyses using C^{13}-labeled substrate, DHA synthesis in *Z. mobilis* is thought to derive from dephosphorylation of DHAP, formed from glyceraldehyde 3-phosphate, an intermediate of the ED pathway [6]. However, no gene encoding the implicated enzyme, dihydroxyacetone phosphatase, has been identified in *Z. mobilis* or in any other microorganism.

Corynebacterium glutamicum, a non-pathogenic high-GC gram-positive bacterium, is wildly used for the industrial production of amino acids [7] and is also emerging as a host organism for the production of fuels [8,9] and chemicals [10–13]. The microorganism produces DHA from sugars (glucose, fructose, and sucrose) under aerobic [14–16] and oxygen-deprived conditions (our unpublished results), but details of the mechanisms involved have not been established. Considering that DHA as a by-product most probably impacts industrial productivity of the microorganism, knowing these details is an avenue to improving *C. glutamicum*-based production processes. This study provides evidence that a previously-uncharacterized member of the haloacid dehalogenase (HAD) superfamily, CgR_2128, in *C. glutamicum* R shows dihydroxyacetone phosphatase activity that is key for DHA biosynthesis from sugars.

Abbreviations: DHA, 1,3-dihydroxyacetone; DHAP, dihydroxyacetone phosphate; HAD, haloacid dehalogenase

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2. Materials and methods

2.1. Microorganisms, cultivation and media

Wild-type *C. glutamicum* strain R (JCM 18229) and its derivatives were grown on a nutrient-rich (A) medium or minimal (BTM) medium with 40 g/l glucose or other sugar [17]. Where appropriate, media were supplemented with kanamycin (50 μg/ml). For analytical purpose, *C. glutamicum* cell starter culture was grown aerobically in A medium at 33 °C overnight. The cells were harvested by centrifugation (5000×*g*, 10 min, 4 °C) and were subsequently washed with minimal medium. The washed cells were inoculated to fresh minimal medium with 40 g/l sugar and were aerobically cultured with agitation (200 rpm).

2.2. DNA manipulations

For overexpression of the *cgR_2128* gene in *C. glutamicum*, the *cgR_2128* gene fragment amplified by PCR with primer 1 and 2 (Supplementary Table S1) was inserted into the NdeI site of a pCRB214 plasmid that is a derivative of an *Escherichia coli–C. glutamicum* shuttle vector pCRB22 [18] and that contains an expression cassette composed of a *tac* promoter, an NdeI site and an *rrn*B T1 terminator. *C. glutamicum* cells were transformed by electroporation [19]. Markerless gene disruption was carried out through a two-step homologous recombination using the suicide vector pCRA725 [8]. For disruption of *cgR_2128*, the nucleotide fragments (ca. 0.9 kb) of 5′- and 3′-regions of *cgR_2128* amplified by PCR with primer set 3–4 and primer set 5–6, respectively (Table S1) were fused by crossover PCR technique. The resulting amplicon was ligated to pCR725, which was subsequently transferred to *C. glutamicum*. Gene disruption was confirmed by PCR and DNA sequencing.

2.3. Enzyme assays

Cell extracts and purified proteins were used for assaying enzyme activities. Cell extracts were prepared with sonication as previously described [19]. Dihydroxyacetone phosphatase activity was measured by quantified inorganic phosphate ion released from phosphorylated organic substrate. Unless otherwise noted, DHAP was used as the phosphorylated organic substrate. Reaction mixture contained (in a final volume of 20 μl) 100 mM Tris–malate (pH7.5), 5.0 mM $MgSO_4$, 5.0 mM DHAP and appropriate amounts of enzyme (0.05–0.5 mg/ml). After 5–10 min reaction, formation of inorganic phosphate was measured using a colorimetric ammonium molybdate-based detection method [20].

2.4. Purification of CgR_2128 protein

The recombinant *C. glutamicum* overproducing *cgR_2128* was grown in A medium for 16 h and subsequently the cells were harvested by centrifugation (5000×*g*, 15 min), and cell lysate was prepared by sonication. The lysate was adjusted to an ammonium sulfate saturation of 35% and the solution was then kept in refrigerator overnight. Precipitate was recovered by centrifugation (6000×*g*, 30 min) and was dissolved in the extraction buffer. Chromatographic separation was carried out using AKTA purifier 10S (GE Healthcare, USA). The sample was applied to a Hiprep 16/10 DEAE FF column (GE Healthcare) equilibrated with buffer A (20 mM Tris–HCl (pH 7.5), 5 mM $MgSO_4$). The adsorbed protein was eluted with a 200-ml linear gradient from 200 to 600 mM NaCl in buffer A. Active fractions were collected and were applied to a MonoQ 5/50 column (GE Healthcare) equilibrated with buffer A. The adsorbed protein was eluted with a 20-ml linear gradient from 300 to 600 mM NaCl in buffer A.

2.5. Analytical techniques

DHA concentration was determined using HPLC (8020, Tosoh, Japan) equipped with a UV detector (210 nm) and an Unison UK-C18 column (Imtakt, Japan) operating at 40 °C with 20 mM phosphoric acid mobile phase at a flow rate of 0.6 ml/min. To determine low concentrations (less than 5 mM) of DHA, GC–MS was used according to the method with modification [21]. Briefly, DHA was acetylated using acetic anhydride with *n*-methylimidazole. 1,3-Propanediol was used as internal standard. Acetylated products were extracted with dichloromethane. The organic phase dried with anhydrous Na_2SO_4 were injected into a GC–MS system (QP2010Plus; Shimadzu, Japan) with a split ratio of 50:1. A capillary column DB-5MS + DG (30 m × 0.25 mm × 0.25 μm; Agilent Technologies, USA) was used for separation. Helium gas was controlled with a constant pressure of 100 kPa. The initial column temperature of 90 °C was hold for 2 min, and then was risen to 240 °C at a rate of 20 °C/min. The inlet and interface temperatures were 210 and 250 °C, respectively. Data acquisition was conducted with the selected-ion monitoring mode. Sugar concentrations were determined using HPLC equipped with an HXP-87P column (BIO-RAD, USA) [19].

3. Results

3.1. A phosphatase-encoding gene is responsible for DHA production in C. glutamicum

Postulating that DHA is produced from the glycolytic intermediate DHAP by the action of a phosphatase, we first searched for genes encoding enzymes that may catalyze this reaction. The fact that no literature on dihydroxyacetone phosphatase-encoding genes was forthcoming drove us to screen an in-house transposon mutant library [22] for strains exhibiting low DHA production. Since both the R and ATCC13032 strains of *C. glutamicum* produce DHA, it is highly likely that identical genes encode enzymes for DHA production in the two strains. Upon comparing the two genomes, 27 putative phosphatase-encoding genes were identified (Supplementary Table S2). Protein phosphatase-encoding genes were not counted in the 27. Single-disruption mutants deficient in 19 of these genes were present in the mutant library. Extracellular DHA production by each of the 19 mutant strains precultured on nutrient rich agar before transferring resultant colonies to minimal liquid medium containing 40 g/l glucose and incubating for 48 h was less than that of wild-type strain (Fig. 1A). The extent of the reduction in mutants' relative DHA yield appeared to closely mirror the reduction in their respective relative growth in all but one or two strains showing the smallest DHA yields (data not shown). Therefore four mutants (deficient in *cgR_1074*, *cgR_1566*, *cgR_2128*, and *cgR_2233*) showing the lowest DHA yields were chosen for further screening. They were cultivated in nutrient rich medium, cells harvested and packed to a density of OD_{610} = 20 (ca. 7.8 mg dry cell weight/ml) in minimal medium containing 40 g/l glucose, and the mixture subsequently shaken for 48 h. Only the strain deficient in *cgR_2128* showed significantly lower DHA yield than wild-type strain (Fig. 1B). We consequently constructed a marker-less mutant deficient in *cgR_2128* (designated as *ΔcgR_2128* strain) to test whether or not the observed reduction in its DHA yield was caused by the polar effect of transposon mutation. In 500 ml flask containing 100 ml of minimal salts medium supplemented with 40 g/l glucose, there was no difference in growth between wild-type strain and *ΔcgR_2128*. DHA production in both strains started after growth entered the stationary phase, but that of the mutant was markedly suppressed (Fig. 2). Both strains showed similar glucose consumption until DHA production started, but after entering the stationary phase glucose consump-

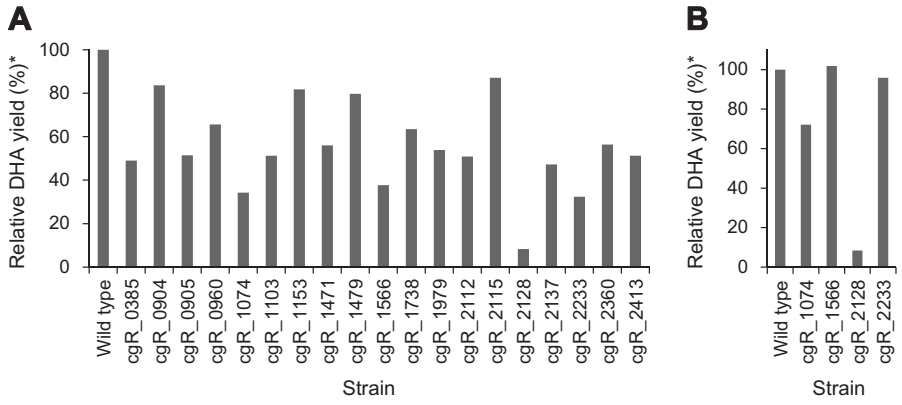

Fig. 1. DHA production of wild-type *C. glutamicum* R and 19 mutants deficient in select putative phosphatase genes. (A) Relative DHA yields of strains cultured in minimal medium containing 40 g/l glucose for 48 h. (B) Relative DHA yields of select strains cultured in complex medium before incubating at high cell density (OD610 of 20) in minimal medium containing glucose for 48 h. The strains were selected by virtue of their exhibiting the lowest DHA productivity when grown on minimal medium. Experimental details are described in the text.

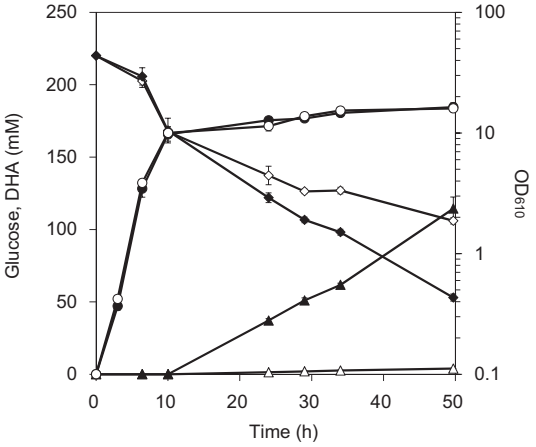

Fig. 2. Growth (circles), glucose consumption (diamonds) and DHA production (triangles) of wild-type (filled symbols) and *cgR_2128* mutant (open symbols) strains grown in minimal medium containing 40 g/l glucose. Data represent means and standard deviations of triplicate experiments.

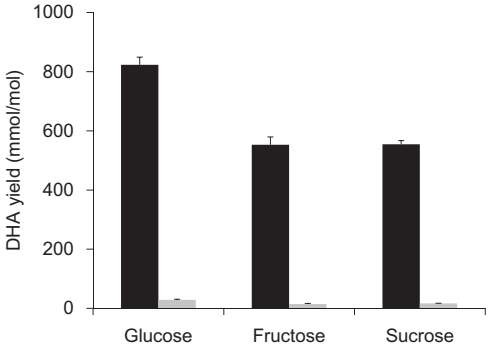

Fig. 3. DHA production of *C. glutamicum* wild-type (darker bars) and *cgR_2128* mutant (lighter bars) strains grown on different sugars. Each strain was cultivated in minimal medium containing 40 g/l sugar for 48 h. DHA yield, expressed as mmol of DHA produced per mol of sugar consumed, was calculated based on the monomeric sugar in the case of sucrose. Data represent means and standard deviations of triplicate experiments.

tion was delayed in the mutant, which seems to be associated with lower DHA production in the mutant. A change of carbon source from glucose to fructose or sucrose similarly decreased DHA production, where DHA yields from glucose, fructose, and sucrose in the mutant were reduced by 3.5%, 2.7% and 3.0% of the control (Fig 3). These results strongly suggest that *C. glutamicum cgR_2128* encodes the enzyme primarily responsible for DHA production during sugar metabolism.

3.2. Plasmid complementation of cgR_2128 mutation restores lost DHA production

The *cgR_2128* gene was overexpressed in the *ΔcgR_2128* strain from a plasmid that constructed from the *C. glutamicum–E. coli* shuttle vector, pCRB22. For the sake of comparison, wild-type and *ΔcgR_2128* strains harboring the plasmid pCRB22 were also constructed. Dihydroxyacetone phosphatase activity in the wild-type strain was too weak to measure, but the same activity in the *cgR_2128*-overexpressing strain was 0.07 ± 0.01 U/mg. As expected, no dihydroxyacetone phosphatase activity was detected in the *ΔcgR_2128* strain. On growing the three recombinant strains in minimal medium containing 40 g/l glucose, the overexpression of *cgR_2128* was confirmed to restore loss of DHA production of the *ΔcgR_2128* strain (Fig. 4).

3.3. cgR_2128 is conserved in C. glutamicum and related taxa

A comparison of nucleotide sequences among four *C. glutamicum* strains (R, ATCC13032, ATCC14067, and S9114) revealed that *cgR_2128* itself as well as genes neighboring it are well conserved among the strains (Supplementary Fig. S1 and Table S3). Homologs of CgR_2128 were also found in wide range of bacteria, especially those in Actinomycetales (Supplementary Fig. S2), though the genetic organization around *cgR_2128* is conserved in only *C. glutamicum* and *Corynebacterium efficiens*, but not in other Actinomycetales listed in Table S4. Protein sequence search showed that CgR_2128 is a member of the HAD superfamily, subfamily IIA (InterPro accession number IPR006357) that includes the *E. coli* NagD protein which catalyzes dephosphorylation of a broad-range of substrates including nucleoside tri- di- and mono-phosphates, particularly CMP and UMP, but not DHAP [23,24].

3.4. Biochemical characterization of CgR_2128

CgR_2128 was purified from *C. glutamicum* harboring the plasmid pCRB22-*cgR_2128* by monitoring dihydroxyacetone phosphatase activity (Table 1). The enzyme sample after the MonoQ separation exhibited a single band in SDS–PAGE and its molecular weight was estimated to be 30 kDa, which is very close to calculated value of 29.5 kDa based on the deduced amino acid sequence

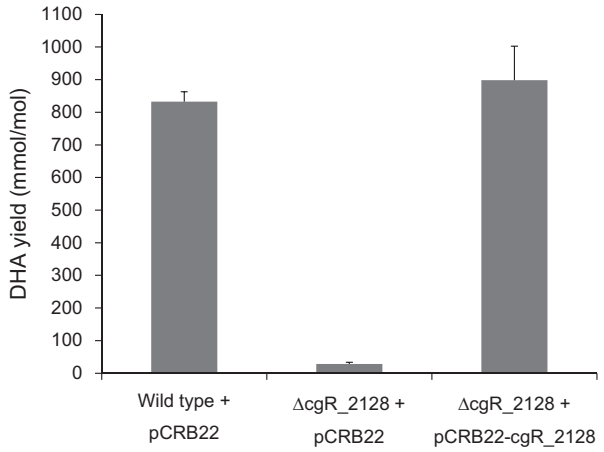

Fig. 4. Complementation of *cgR_2128* mutation by overexpression of *cgR_2128*. A control plasmid (pCRB22) or a *cgR_2128*-overexpressing plasmid (pCRB22-*cgR_2128*) was used to transform wild-type or *cgR_2128* mutant strains. Recombinant strains were cultivated in minimal medium containing 40 g/l glucose for 48 h. DHA yield was expressed as mmol of DHA produced per mol of glucose consumed. Data represent means and standard deviations of triplicate experiments.

Table 1
Purification of dihydroxyacetone phosphatase.

	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Purification (fold)
Cell free extract	121	8.5	0.07	1
$(NH_4)_2SO_4$ ppt	58	4.5	0.08	1.1
DEAE	4.7	2.6	0.56	8
MonoQ	1.7	1.8	1.05	15

of CgR_2128. The N-terminal amino acid sequence of the purified enzyme was determined to be TVNIS, which corresponds to the deduced amino acid sequence of CgR_2128 after the N-terminal methionine (Met). Since the amino-terminal Met is often processed by methionine aminopeptidase in *C. glutamicum* [25], we concluded that the enzyme purified by monitoring of the dihydroxyacetone phosphatase activity is CgR_2128. To assess the ternary structure of CgR_2128, the purified enzyme was subjected to gel filtration chromatography and a molecular weight of 180 kDa estimated (data not shown), suggesting that CgR_2128 formed homohexamer.

Although purified CgR_2128 showed higher activity in lower pH range within 5.5 to 8.0, enzyme characterization had to be done at pH 7.5 in this study. As is the case with other phosphatases belonging to the HAD superfamily, Mg(II) enhanced the activity. Stoichiometry of the enzymatic reaction was determined by measuring inorganic phosphate (Pi) using a malachite green assay and DHA using HPLC. The ratio of Pi to DHA so determined was 0.98. No activity toward nucleoside monophosphates (AMP, CMP, GMP, or UMP) at a concentration of 5 and 10 mM was detected. When steady-state kinetic analysis of CgR_2128 was performed, initial velocity of the enzyme reaction increased with increasing DHAP concentration, but no saturation was observed at DHAP concentrations up to 38 mM.

4. Discussion

Here we demonstrated that *cgR_2128* encodes a HAD superfamily protein with dihydroxyacetone phosphatase activity that is central to DHA formation during sugar metabolism in *C. glutamicum*. Because there are 26 genes for HAD proteins in the genome of *C. glutamicum* R, we designate *cgR_2128* the *hdpA* (HAD superfamily phosphatase) gene.

Formation of DHA by *C. glutamicum* has been described especially in lysine production by engineered strains [14,16,26–29]. DHA formation by *C. glutamicum* has been speculated to be the result of overflow metabolism caused by accumulation of glycolytic intermediates upstream of glyceraldehyde 3-phosphate dehydrogenase [15,28,29]. In this study, *C. glutamicum* HdpA did not show typical Michaelis–Menten type saturation kinetics even when DHAP concentration was increased to 38 mM, suggesting that substrate affinity of HdpA to DHAP is relatively low. This characteristic of HdpA seems to be consistent with the hypothesis of the overflow metabolism; DHA is formed by the action of HdpA only when excess DHAP accumulates in cells. Although this study revealed the fundamentals of DHA biosynthesis in *C. glutamicum*, further studies are required to understand regulation of the DHA biosynthesis.

Protein similarity searches confirmed HdpA to belong to the HAD superfamily that permeates all three domains of life (InterPro database). Despite the naming, the majority of characterized enzymes in this superfamily are phosphatases with several *P*-type ATPases, dehalogenases, and β-phosphoglucomutases [30]. These disparate functions make it difficult to elucidate functions of HAD proteins based solely on sequence similarity. The InterPro database [31] classifies HdpA as a member of subfamily IIA that includes *E. coli* NagD and *Bacillus subtilis* AraL. Biochemical characterization of the latter two has revealed phosphatase activities toward various substrates including nucleotides (NagD) and sugar phosphates (AraL), but not DHAP [23,24,32]. This ascertains the difficulty in predicting protein function of HAD proteins from sequence similarity even at subfamily level. The *E. coli* genome encodes 23 soluble HAD hydrolases, 21 of which show phosphatase activity with broad and overlapping substrate profiles [24]. This, combined with the low affinity of HdpA to DHAP, suggests that HdpA might be active on other phosphorylated metabolites and have multiple physiological roles.

Although dihydroxyacetone phosphatase activity has been detected in *Z. mobilis* [6], no homolog of *C. glutamicum* HdpA was found in the *Z. mobilis* genome, implying that a structurally different dihydroxyacetone phosphatase(s) evolutionary unrelated to *C. glutamicum* HdpA may be present in *Z. mobilis*.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.febslet.2012.10.028.

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